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A CALCIUM DEPENDENT PROTEIN KINASE INVOLVES H_2O_2 MEDIATED GUARD CELL SIGNALING IN *ARABIDOPSIS*

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ABSTRACT

Drought is a major threat for plant growth and productivity. Plants lose over 90% of water by transpiration through stomatal pores. The cytosolic free Ca^{2+} elevated in guard cells in response to stress stimuli triggers stomatal closure. The plant-specific calcium-dependent protein kinases (CDPKs) play important roles in regulating downstream components of calcium signaling. In this study the biological function of *Arabidopsis* calcium-dependent protein kinase, *CPK8,* in response to ABA signaling in guard cells was characterized. The plants of T-DNA insertion mutant of *cpk8* were more sensitive to drought stress than wild-type plants. The GUS staining studies confirmed that *CPK8* expressed in leaves, specifically in guard cells. RT-PCR analysis showed that *CPK8* expression was induced in response to drought stress. Further, pre-opened *cpk8* stomata failed to close in response to H_2O_2 and Ca^{2+}, which is consistent with the inability of *cpk8* plants to reduce water loss upon drought. The drought susceptibility and stomatal impairment in response to H_2O_2 and Ca^{2+} of the *cpk8* implicated that *CPK8* plays a role in cellular environment in the control of H_2O_2 homeostasis and also as a compulsory molecule in the transduction of H_2O_2 signal in guard cells in response to drought stress.

Key words: *Arabidopsis thaliana*, Calcium-dependent protein kinases (CDPKs)

INTRODUCTION

Water is the most limiting resource for terrestrial plant growth and development and yield formation in many part of the world. Desiccation of crops during various growth stages causes severe and often irreversible damage and hence yields losses (Boyer 1982; Ainsworth and Long 2005). It would be beneficial for crop plants to show wide stomatal opening for CO_2 intake when water is available, but to close stomata during drought periods, thereby slowing desiccation and damage. Stomatal opening is driven by plasma membrane hyper polarization proposed to drive K^+ uptake into guard cells passively via inward-rectifying K^+ (K^+_{in}) channels (Schroeder *et. al.* 1987). Stomatal closing involves the influx of free cytosolic Ca^{2+} that down regulate the inward K^+ channels and activate outward channels (Li *et al*. 2000). Ca^{2+} oscillations are a fundamental requirement for stomatal closure.

Several classes of Ca^{2+} binding sensory proteins have been identified in plants. CDPKs are the largest subfamilies of plant protein kinases among them. The completed *Arabidopsis* genome sequence has revealed 34 genes encoding CDPKs and they are highly homologous to each other (Cheng *et al*. 2002; Hrabak *et al*. 2003). Recently genome wide analysis of rice found that there are 29 genes encoding CDPKs and eight closely related kinase genes (Takayuki *et al*. 2005). Some other plants including soybean, tomato and maize also indicate the presence of multi-gene families (Harmon *et al*. 2001), but the reason for such a large number of CDPK genes is not yet known. Recent experiments indicate that functional specialization of individual CDPKs can occur through different types of regulation. For example, plants may use a combination of various strategies to functionally specialize individual CDPKs, as evidenced by two sandalwood CDPK isoforms that differ in tissue specific distribution, sub-cellular localization, and enzyme kinetics and properties (Anil *et al*. 2001).

CDPKs play divers roles in various biological responses by interacting with other factors and act as key regulators of many signaling path-

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ways. But very little is known about the particular CDPK that acts as the calcium sensor in each case. In this study we characterize the biological function of *Arabidopsis* calcium-dependent protein kinase *CPK8* in response to ABA signaling in guard cells.

MATERIALS AND METHODS

Plant Materials and Growth Conditions: *Arabidopsis thaliana* ecotype Columbia was used in this study. The T-DNA insertion mutants of *CPK8* (*cpk8*, SALK_036581) was obtained from Arabidopsis Biological Resource Center (ABRC; http://www.arabidopsis.org/abrc/). The homozygous *cpk8* mutant was identified using gene-specific primers (forward primer 5′-CATGTTTGCGTTTGTGAGTG-3′ reverse primer 5′-GGCTTTAAGGGCTGAT GTC-3′).

Seeds were sterilized using NaOCl solution (0.5% NaOCl and 0.01% Triton x-100) for 10-15 min. and washed 5 times with sterilized distilled water under aseptic conditions in laminar flow cabinet and kept in dark at 4℃ for 72 hours to break the dormancy.

Sterilized seeds were sown on MS plates and incubated in 20-22℃ with 120 μ/mol/m^2/s light intensity for seedling development. Seven day old seedlings were then transferred to 1:1 soil: peat medium. After transplanting, plants were covered with polythene cover to maintain high humidity and kept in growth chambers at 22℃ with illumination at 120 μ/mol/ m^2/ s for 16-h light/8-h dark cycle. The relative humidity was approximately 70% (±5%). One week later plants were disclosed.

Vector Constructions and Generation of Transgenic Plants: The $CPK8_{pro}$*:GUS* construct was generated by fusing the *CPK8* promoter fragment (1.96 kb) in front of the β-glucuronidase (GUS) coding sequence in pCAMBIA1381 vector. The special primers for $CPK8_{pro}$*:GUS* construct were 5’-CACTCTCCTAGGAACCGATAC-3’ and 5’-TTCGAATCTGAGAAGTCCTG-3’. The GUS staining assays were carried out as described by (Xu et al. 2006).

Drought tolerance measurements: For drought experiments, *Arabidopsis thaliana* [Columbia ecotype, *cpk8* knockout mutant] plants were grown on MS medium under continuous light for 7 days and transferred to peat soil in a controlled environment growth chamber with a 16:8 hour light: dark cycle and irrigated for 2 weeks. Then plants were subjected to drought by complete termination of irrigation. The plants (n=12 each) at the similar developmental stages were selected for the analysis. Watered plants were analyzed as control treatment. Pots were weighed after 3, 6, 9, 12, 15 and 18 days at the same time for relative water content measurements.

Water loss Measurements: Water loss experiments were conducted on weight basis and at the same time phenotype comparison at different time intervals were carried out. For measurement of water loss, plants were transferred from high (90%) to low humidity (50%) and then leaves were detached and incubated abaxial face up in 25℃with 50% RH, Their fresh weight was measured at different time intervals. Water loss was expressed as the percentage of initial fresh weight. To compare the phenotype, photographs were taken just after detaching and 5 hours after desiccation. Each experiment repeated 6 times with 4 replicates.

Stomatal aperture measurements: Plants were grown on MS medium under continuous light for 7 days and transferred to peat soil in a controlled environment growth chamber with a 12:12 hour light: dark cycle for 3 weeks and then placed in overnight dark before every treatment. To measure stomatal opening, detached leaves were floated in incubation buffer containing 50mM KCl with10mM MES/KOH and 0.1mM $CaCl_2$ for 3 hours under light to induce stomatal opening and measured the aperture width.

To measure the stomatal closing, detached leaves were floated in incubation buffer under

light for 2 hours and then kept in dark for another 2 hours to induce stomatal closing. After 2 hours, stomatal apertures were measured. For ABA-inhibition of stomatal opening, leaves were incubated with or without ABA (10 µM) under light for 2.5 hours. To study the effect of H_2O_2 and Ca^{2+}, stomata were opened by exposing plants for light and high humidity and incubating the leaves for 2 h in stomata-opening solution containing 50 mM KCl, 0.1 mM $CaCl_2$, and 10 mM MES/KOH, pH 6.15, in a growth chamber at 22 to 25^{0}C under a photon flux density of 0.20 to 0.30 mmolm^{-2}s^{-1}. Stomatal apertures were measured 2 h after adding 100 µM H_2O_2 or 5mM Ca^{2+}.

RESULTS

Phenotype Characterization of *CPK8* T-DNA Insertion Mutant and Expression Patterns of *CPK8:* No obvious morphological difference was observed between the *cpk8* and wild-type, columbia plants under normal growth conditions (Fig. 1A). Fourteen days after withholding water, *cpk8* showed severe wilting symptoms compared to wild type and re-watering did not allow the complete recovery of *cpk8* plants (Fig. 1A). The site of T-DNA insertion in *cpk8* was verified by conducting reverse transcription (RT)-PCR experiments (Fig.1B). RT-PCR analysis showed that there is no transcript of *CPK8 in cpk8* homozygous plants (Fig. 1C), suggesting that *CPK8* expression is completely eliminated in *cpk8* mutant. To investigate the expression patterns of *CPK8*, transgenic plants harboring a *GUS* reporter gene fusing with *CPK8* promoter was generated. High GUS activities were detected in the leaves and abundantly in stomatal guard cells, suggesting the potential role of CPK8 in regulation of stomatal movement (Fig. 1D).

Drought Sensitivity: When grown under well -watered conditions, the *CPK8* T-DNA mu-

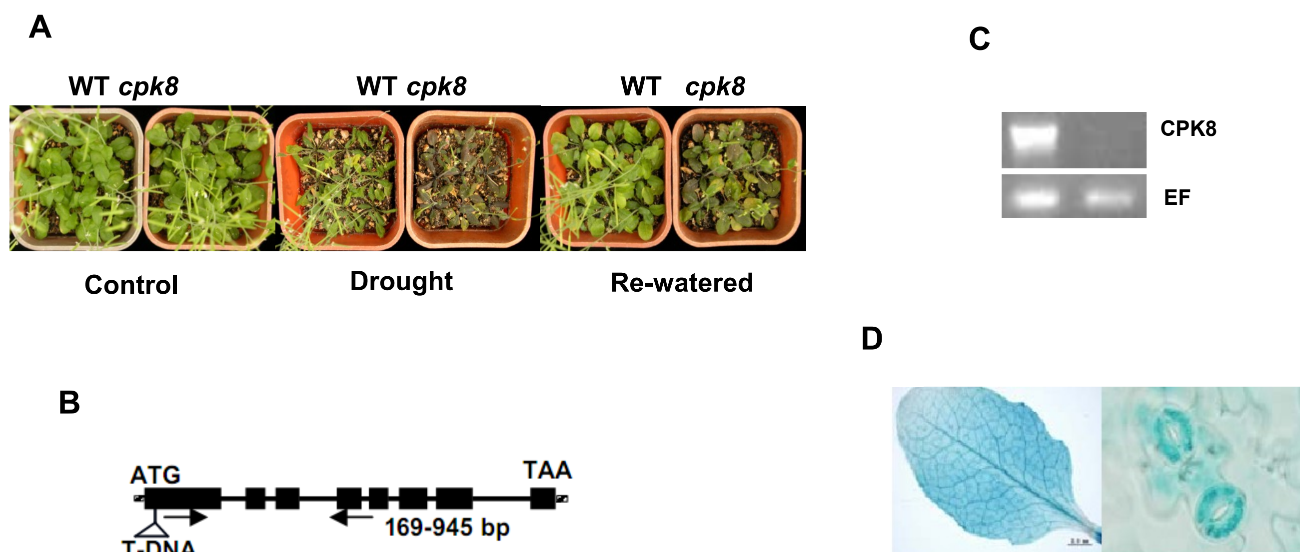

Figure 1: Characterization of *cpk8* T-DNA insertion mutant in response to drought.
A-Phenotypic comparison between wild-type (WT) *Arabidopsis* (ecotype Columbia) and *cpk8* during drought t reatment
B-T-DNA insertion site in the *cpk8* mutant. The T-DNA was inserted in the first exon of the *CPK8* genomic DNA. Black boxes, solid line and diagonal boxes denote exons, introns and untranslated regions, respectively. Solid arrows indicate the primer locations for *CPK8* kinase domain transcript.
C-RT-PCR verification of *CPK8* expression in *cpk8*. Elongation factor 1a (EF) was used as loading control.
D-Expression patterns of *CPK8* as determined by *CPK8:GUS* transgenic plants. Transgenic plants were stained with 5- bromo -4- chloro -3- indolyl -β- D-glucuronic acid solution for 12 hours Gus staining is shown in rosette leaves and guard cells.

tant plants did not display any visible phenotypic alteration. First, plants were grown with optimum irrigation up to two weeks and subjected to water stress by complete termination of watering. Fourteen days after withholding water, *CPK8* T-DNA mutant showed severe wilting symptoms compared to wild type. Re-watering did not allow the complete recovery of *CPK8* T-DNA mutant compared to wild type (Fig. 1A). RT-PCR verification of *CPK8* expression in *cpk8* is shown in figure 1C.

Relative water content (RWC) is a good indicator of a plant water status at any given time because it closely reflects the balance between water supply and transpiration rate. RWC were determined by weighing pots and expressing the weight loss as a percentage of initial fresh weight during desiccation. Transpirational water loss, as determined by RWC measurements after 3 days from the start of the treatment, was greatly decreased in the mutant compared to the wild type lines upon drought treatment (Fig 2).

Water Loss Measurements of Detached Leaves: Water loss from detached wild-type, and *cpk8* mutant rosette leaves were measured during incubation at 25℃ and 50% relative humidity. This experiment repeats 5 times each with 4 replicates. After 3 hours of desiccation *cpk8* leaves displayed wilting symptoms and wild type leaves still remain turgid. By 5 hours after treatment mutant leaves completely wilted due to dehydration and wild type line still remained turgid (Fig. 3A). The fresh weight of detached leaves was measured at hourly time intervals. Throughout the duration of the desiccation treatment, mutant leaves consistently lost higher amount water than wild-type leaves (Fig.3B).

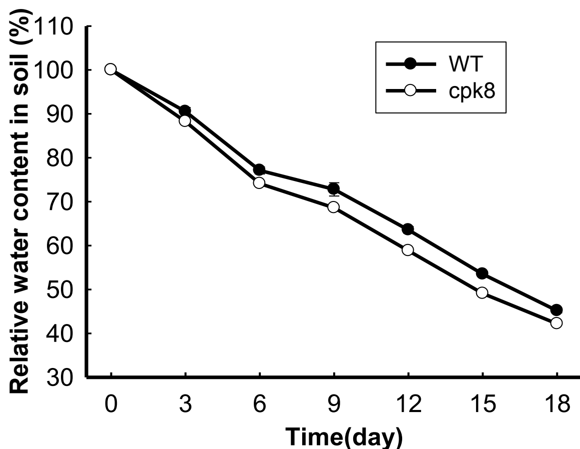

Figure 2: Comparison of relative water content (RWC) between *cpk8* and wild type (ecotype Columbia) plants

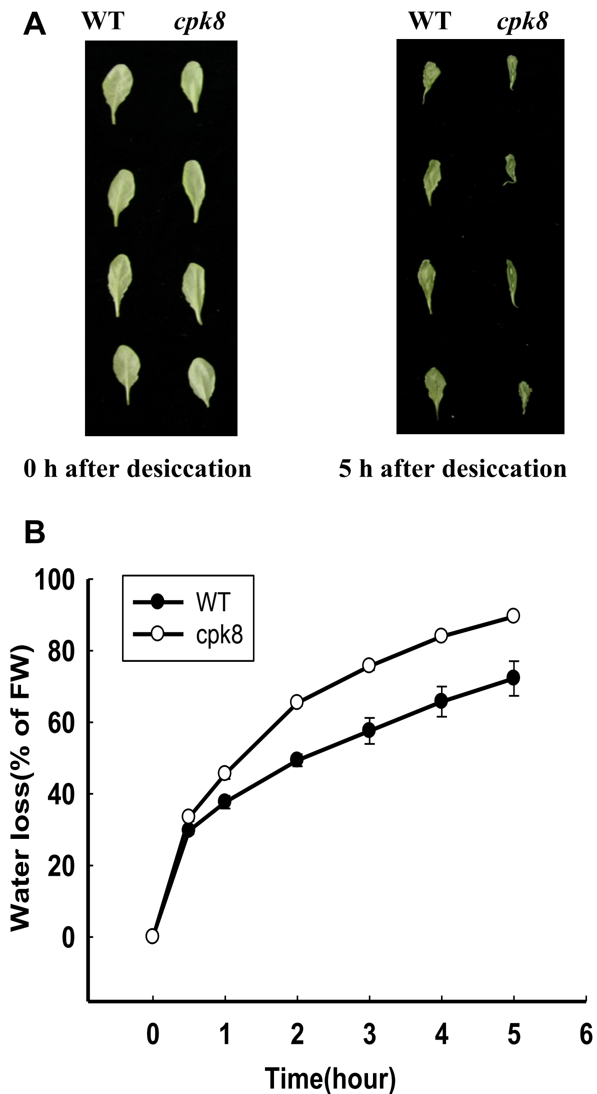

Figure 3: Comparison of water loss measurements between *cpk8* and wild type (ecotype Columbia) plants

(A). Phenotype comparison of water loss of detached leaves, at the beginning and 5 hours after desiccation.

(B). Time course of water loss from excised leaves, expressed as a percentage of initial fresh weight.

Stomatal Aperture Measurements: To explore whether drought sensitivity observed for the *cpk8* plants correlates with stomata performance, stomatal apertures were measured with different treatments. The *cpk8* stomata closed to the same extent as the wild type in response to darkness (Fig. 4A). Similarly, the *cpk8* mutations had no effect on the ability of pre-closed stomata to open in response to light (Fig. 4A).

It is well characterized that plants typically synthesize ABA in response to drought, which triggers the closing of stomata, thus reducing water loss and enhancing drought stress resistance (Schroeder *et al*., 2001; Luan, 2002). We tested whether the gene disruption affects the stomatal movements in the mutant treated with ABA. Leaf materials were incubated in10μM ABA under light. ABA induced stomatal closure partially impaired in comparison to wild type (Fig. 4B).

The exogenous H_2O_2 induced elevations of cytosolic calcium and stomatal closure (Pei *et al*. 2000). However, preopened *cpk8* stomata failed to close in response to H_2O_2 (Figure 5A), which is consistent with the inability of *cpk8* plants to reduce water loss upon drought.

Further, CDPKs have been predicted to function in response to cytoplasmic Ca^{2+} elevations in many physiological processes in plants. Extracellular Ca^{2+} causes stomatal closing, by initiating repetitive cytoplasmic Ca^{2+} elevations in guard cells (Pei et al. 2000). To investigate the role of *CPK8* in guard cell signaling, detached leaves were incubated in 5mM $CaCl_2$. Addition of $[Ca^{2+}]_{ext}$ to pre-opened wild-type stomata caused closure, whereas in *cpk8* stomatal closure was significantly attenuated suggesting that CDPKs function in $[Ca^{2+}]_{cyt}$ perception and ion channel activation (Fig. 5B). These data indicate that the *cpk8* mutations do not cause a general defect in stomatal functioning but specifically disrupt H_2O_2 and Ca^{2+} signaling in guard cells.

Excess H_2O_2 Accumulation in the *cpk8* Mutant under Drought Stress: As an important signaling molecule, H_2O_2 had been identified to mediate ABA signal transduction in stomatal guard cells (Pei *et al*., 2000; Murata *et al*. 2001; Zhang *et al*. 2001b; Kwak *et al*. 2003; Bright *et al*. 2006; Miao *et al*. 2006; Yan *et al*. 2007). It is known that excess ROS (Reactive Oxygen Species) accumulation in living plant cells is toxic to cellular activities,

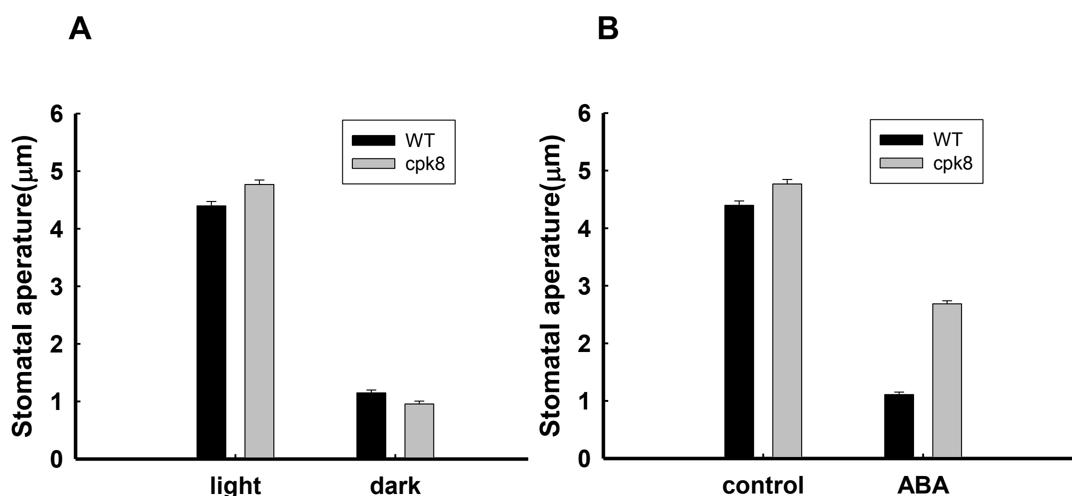

Figure 4: Light and Dark induced Stomata regulation and ABA-inhibition of stomatal opening
A. Average data for 3 representative experiments from each treatment are shown, n=150 total stomata).
B. Stomatal apertures were determined for wild-type and *cpk8* mutant plants treated with10μM ABA. Data were averaged across 4 separate experiments;
***n* = 50 aperture measurements per experiment. Error bars represent standard errors.**

so the cytosolic concentration of ROS must be stringently controlled (Mittler 2002; Apel and Hirt 2004). To test if H_2O_2 accumulation would be changed in *cpk8* mutants, 3, 3'-diaminobenzidine (DAB) uptake method (Thordal-Christensen *et al*. 1997; Guan and Scandalios 2000) was applied to examine the production of H_2O_2 in leaves of *cpk8*, and wild-type plants. As shown in Fig. 6, ABA-induced H_2O_2 accumulation was significantly increased in the leaves of *cpk8* mutants compared to wild-type plants. This demonstrated that CPK8 plays an important role in H_2O_2 homeostasis.

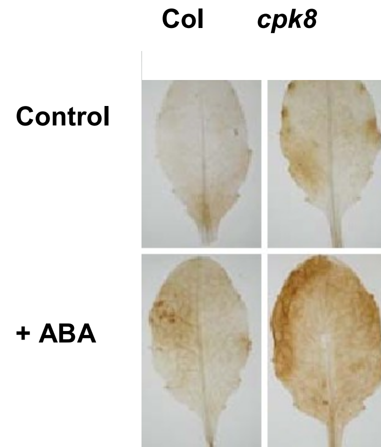

Figure 6: Comparison of ABA-induced H_2O_2 production in leaves of *cpk8* and wild type ABA-induced H_2O_2 production in leaves of wild-type plants and *cpk8* mutant assayed with 3,3-diaminobenzidine (DAB). The experiments were repeated for five times with similar results.

DISCUSSION

CDPKs have been found to function in response to cytoplasmic Ca^{2+} elevations in many physiological processes in plants (Harmon *et.al*. 2000). Evidences for a role of CDPKs in biotic stress signaling and environmental stress signaling were previously reported (Romeis 2001, Sheen 1996, Saij, 2000). The potential of CDPKs for engineering useful traits has also been suggested by alterations in the expression of rice OsCDPK7 that influenced cold and salt/drought tolerance in transgenic rice plants (Saijo 2000). Results of this study provided that AtCDPK8 plays a role in the transduction of an H_2O_2 signal in guard cells that mediates stomatal regulation in response to drought stress.

In addition, very strong expression of ATCDPK8 promoter GUS in transgenic plants was observed in guard cells of the leaf epidermis (Figure 1D), implying that ATCDPK8 specifically functions in guard cells. Stomatal

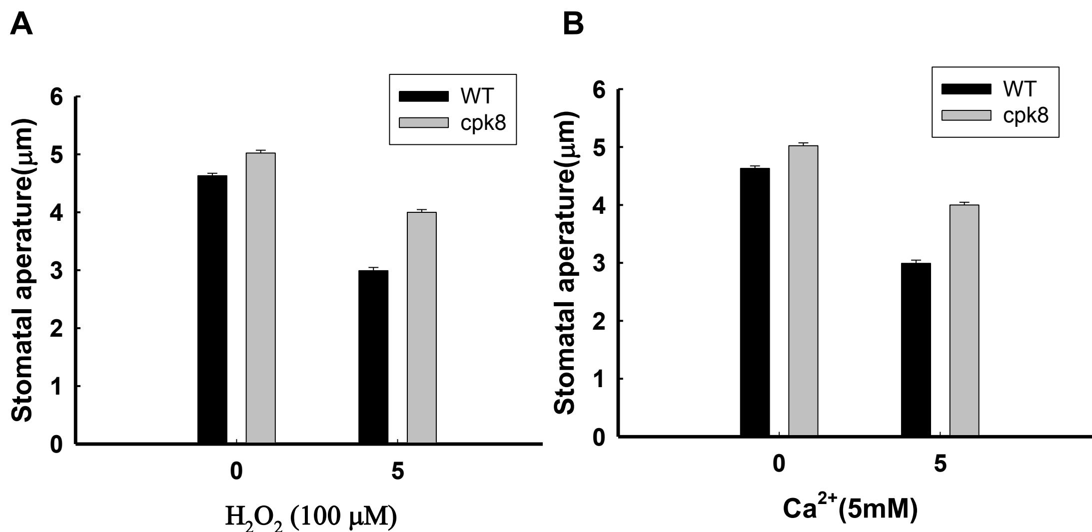

Figure 5: H_2O_2 and Ca^{2+}-induced stomatal closure is impaired in *cpk8* mutant.
(A) H_2O_2-induced stomatal closing. (Average data from 3 representative experiments, n=150 total stomata).
(B) External Ca^{2+}-induced stomatal closing. (Average data from 3 representative experiments, n=150 total stomata).

aperture measurements showed that stomata from wild-type and AtCDPK8 plants closed to the same extent in the dark and after exposure to light, both materials displayed same extent in the opening of stomatal pores (Fig.4A). Pei *et al* (2000) reports that H_2O_2 induced elevations of cytosolic calcium and stomatal closure. However, pre-opened *cpk8* stomata failed to close in response to H_2O_2 and Ca^{2+} (Figure 5A, 5B), which is consistent with the inability of *cpk8* plants to reduce water loss upon drought suggesting that loss-of-function mutation in *CPK8* impairs H_2O_2 homeostasis and signal transduction in guard cells. Hence, it is possible that CPK8 acts as a key regulator that specifically modulates H_2O_2 homeostasis and as an essential signal transduction molecule in guard cell signal transduction pathway. These data indicate that the *cpk8* mutations do not cause a general defect in stomatal functioning but specifically disrupt H_2O_2 signaling in guard cells. Recent reports revealed that ABA- and Ca^{2+}-induced stomatal closing were partially impaired in cpk3 cpk6 mutant alleles (Mori *et al*. 2006). Similarly, stomatal closure in *AtCDPK8* recessive plants was impaired in response to Ca^{2+} and H_2O_2 but not to ABA whereas closure elicited by darkness and opening elicited by light were unaffected suggesting an alternate signaling pathway. On the basis of the results presented here as well as those reported previously (Mori *et al*. 2006; Pei *et al*. 2000), allows to describe a divergent oxidative signal transduction pathway from sensing to the drought response in plants.

CONCLUSION

Results of this study confirmed the potential location of the AtCDPK8 by GUS staining and RT-PCR verified the presence and absence of AtCDPK8 in transgenic knockout mutants and wild type plants respectively. Phenotypic expression along with stomatal and DAB staining studies revealed that AtCDPK8 plays a role in the transduction of an H_2O_2 signal in guard cells mediating stomatal regulation in response to drought stress via divergent oxidative signal transduction pathway.

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